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(71) Applicant *(for all designated States except US)*: **UNIVERSITE LIBRE DE BRUXELLES** [BE/BE]; Avenue F.D. Roosevelt 50, CP 165, B-1050 Brussels (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **VAN LINT, Carine** [BE/BE]; Corniche Verte 16, B-1150 Brussels (BE). **BURNY, Arsène** [BE/BE]; Chaussée de Namur 65, B-5030 Gembloux (BE). **QUIVY, Vincent** [BE/BE]; Rue de l'Ardoisière 18, B-1430 Rebecq (BE). **ADAM, Emmanuelle** [BE/BE]; Le Croquet 3, B-5340 Faulx-Les-Tombes (BE).

(74) Agents: **VAN MALDEREN, Joëlle** et al.; Office Van Malderen, Place Reine Fabiola 6/1, B-1083 Brussels (BE).

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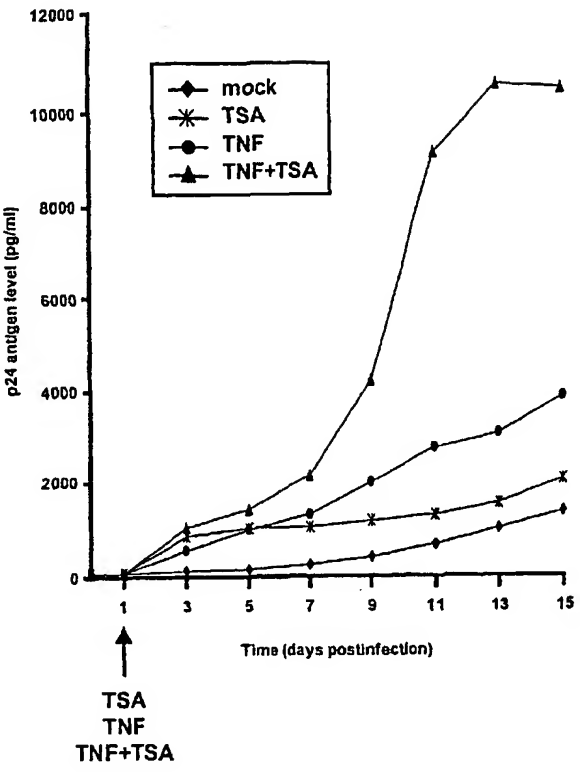
(54) Title: METHOD FOR OBTAINING THE ELIMINATION OF INTEGRATED AND FUNCTIONAL VIRUSES FROM INFECTED MAMMAL CELLS



(57) Abstract: Use of a sufficient amount of deacetylase inhibitor combined with one or more compounds used in a viral treatment for the manufacture of a medicament for obtaining the elimination of integrated, functional and pathogenous viruses in a mammal cell, including a human cell.

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METHOD FOR OBTAINING THE ELIMINATION OF INTEGRATED AND FUNCTIONAL VIRUSES FROM INFECTED MAMMAL CELLS

Field of the invention

[0001] The present invention is related to a method for obtaining the elimination of integrated and functional viruses, especially HIV viruses, from infected mammal cells preferably with the combination with a continuous HAART (Highly Active Antiretroviral Therapy) treatment for obtaining a significant elimination and possibly suppression of the viral presence in a mammal patient and therefore for improving long term control of viral infections in infected mammals receiving HAART treatment.

Background of the invention

[0002] The persistence of latently HIV-infected cellular reservoirs, despite prolonged treatment with HAART (Highly Active Antiretroviral Therapy), represents the major documented hurdle to virus eradication. These latently infected cells are a permanent source for reactivation and lead to a rebound of viral load levels after interruption of HAART (reviewed in (Pierson et al., 2000). Therefore, a greater understanding of the molecular mechanisms regulating viral latency and reactivation should lead to rational strategies aimed at purging the latent HIV reservoirs (Ho, 1998; Cohen, 1998). At the cellular level, two major forms of HIV-1 latency have been described: pre-integration latency and post-integration latency (reviewed

in (Pomerantz et al., 1992). Several cell lines selected *in vitro* have served as models for studying this latter type of latency. Production of viral particles can be induced in these cell lines at the transcriptional level by a variety of agents, including phorbol esters and cytokine TNF (Pomerantz et al., 1990). Several explanations have been proposed for the low level of transcription observed during post-integration latency, they include:

1) the site of integration of the provirus into the host cell genome and the cellular chromatin environment at this site (Jordan et al., 2001).
2) the absence of the viral trans-activator Tat, which binds to TAR, a RNA hairpin loop formed at the 5' termini of all nascent HIV-1 transcripts (Adams et al., 1994).
3) the presence of mutations in the integrated provirus, including interruption of the Tat-TAR axis (Emiliani et al., 1998).
4) the presence of a potentially repressive nucleosome (nuc-1) located immediately downstream of the HIV transcription start under latency conditions. Nuc-1 is remodeled upon activation of the HIV promoter located in its 5' Long Terminal Repeat (LTR) in response to Tat, phorbol esters and deacetylase inhibitors (Van Lint et al., 1996a).
5) The absence of NF-KB.

[0003] The enhancer region in the U3 region of the LTR contains two binding sites for the inducible transcription factors NF-KB, which plays a central role in the activation pathway of the HIV-1 provirus (reviewed in Rabson and Lin, 2000). Various studies have reported that NF-KB-binding sites (Kim et al., 1993) as well as the NF-

KB proteins (Quian et al., 1994) are critical for LTR promoter activity and important for optimal HIV-1 replication (Duckett et al., 1993). NF-KB is an inducible transcription factor complex that plays a role in the expression of a variety of genes involved in immune and inflammatory responses and cell survival reviewed in (Karin and Ben Neriah, 2000). In mammalian cells, there are five known members of the NF-KB/Rel family: p65 (RelA), c-Rel, RelB, p50 (NF-κB1), and p52 (p49, NF-κB2). The most widely studied and most abundant form of NF-KB is a heterodimer of p50 and p65. In unstimulated cells, NF-KB is sequestered in the cytoplasm in an inactive form through interaction with members of the inhibitor κB (IκB) family of proteins including IκB-alpha, IκB-beta and IκB-epsilon. Upon activation of NF-KB by various stimuli (including inflammatory cytokines (TNF, IL-1), bacterial toxins (such as lipopolysaccharides), viral proteins, mitogens (phorbol esters), UV light), IκBs are rapidly phosphorylated by a macromolecular IκB kinase complex (IKK) (Israel, 2000) ubiquinated and degradated by the 26S proteasome. The released NF-KB then translocates to the nucleus, where it can activate transcription from a wide variety of promoters, including that of its own inhibitor IκB-alpha (see for review (Karin and Ben Neriah, 2000)). After NF-KB-dependent resynthesis, IκB-alpha enters the nucleus, enhances NF-KB removal from DNA, and takes it back to the cytoplasm, thus restoring the inducible cytoplasmic pool of NF-KB. Thus, the *de novo* expression of IκB-alpha proteins, which display nucleocytoplasmic shuttling properties, participates in a negative feedback system ensuring a

transient NF-KB transcriptional response (Arenzana-Seisdedos et al., 1997).

[0004] There is now strong evidence that both transcriptional activation and silencing are mediated through the recruitment of enzymes that control protein acetylation. Acetylation of specific lysine residues within nucleosomal histones is closely linked to chromatin disruption and transcriptional activation in many genes (reviewed in (Roth and Allis, 1996)). Consistent with their role in altering chromatin structure, many transcriptional coactivators (including GCN5, CBP/p300, P/CAF, SRC-1) possess intrinsic histone acetyltransferase (HAT) activity that is critical for their function (Roth et al., 2001). Similarly, corepressor complexes include proteins that have histone deacetylase (HDAC) activity (reviewed in (Khochbin et al., 2001)). Importantly, reversible acetylation is also a critical posttranslational modification of non-histone proteins, including general and specific transcription factors, coactivators, non-histone structural chromosomal proteins, and nuclear import factors. Protein acetylation regulates many diverse functions, including DNA binding, protein/protein interaction, protein stability and cellular localization (see for review (Chen et al., 2001a)). Hence, acetylation may rival phosphorylation as a mechanism for the transduction of cellular regulatory signals.

[0005] In the case of HIV-1, ample evidence reports that viral transcription is regulated by acetylation of histones and non-histones proteins. Transcriptional activation of the HIV-1 promoter in response to TSA has also been demonstrated in *ex vivo* transiently or stably transfected HIV LTR reporter constructs (Jordan et al., 2001) and on *in vitro* chromatin-reconstituted HIV-1 templates (Steger et al., 1998). Moreover, acetylation of Tat by p300, by P/CAF

and by hGCN5 is important for its transcriptional activity (Col et al., 2001). The LTR also contains several binding sites for transcription factors, which have been shown either to be directly acetylated or to interact with deacetylases and/or acetyltransferases.

Summary of the invention

[0006] The present invention is related to a method for obtaining the elimination of integrated and functional viruses, especially retroviruses such as HIV viruses, from infected mammal cells, including human cells. Preferably said method is combined with a viral treatment especially HAART (Highly Active Anti-Retroviral Therapy) for obtaining a significant elimination of virus' cellular reservoirs and therefore for improving longterm control or erradication of viruses in infected mammals, including humans, especially mammals infected by HIV and receiving HAART treatment.

[0007] Therefore, a first aspect of the present invention is related to the use of a sufficient amount of deacetylase inhibitor(s) combined with one or more compounds used in classical viral treatment of a mammal patient, including human patients in the manufacture of a medicament for the elimination of integrated and functional viruses in patient cells. Preferabaly, said viral treatment is HAART (Highly Active Anti-retroviral Therapy) treatment which is a combination of several known or unknown antiviral compounds effective to treat HIV infections (see review by Kuan-The-Jeang, HIV-1 Molecular Biology and Pathogenesis Advances in Pharmacology, Vo..49, Academic press, San Diego, (ISBM 012-032950-6)).

[0008] Therefore, said medicament could be used in the treatment and/ or the prevention of AIDS (syndrome induced by HIV-1 and/ or HIV-2 virus infections).

[0009] Therefore, the present invention is also related to a method of treatment and/ or prevention of viral infections, especially retroviruses infections such as HIV-1 and or HIV-2 viruses infections in a mammal patient, including human patients (AIDS). Said method of treatment and/ or prevention comprising the step of administrating to said mammal patient (including a human patient), suffering from said viral infection, a sufficient amount of deacetylase inhibitor(s) combined with one or more compounds used in HAART treatment used to obtain the elimination of integrated and functional viruses in a mammal cell of said mammal patient.

[0010] Said method of elimination could be obtained by in vivo treatment or ex vivo treatment when said method is applied upon a biological fluid (blood) obtained from said mammal patient.

[0011] Preferably, the inhibitor of the deacetylase(s) is combined with the antiviral compound(s) used in HAART treatment in an adequate pharmaceutical carrier or diluant.

[0012] Another aspect of the present invention is related to a pharmaceutical composition comprising an adequate pharmaceutical carrier or diluant and a deacetylase inhibitor combined with one or more antiviral compounds used in HAART treatment.

[0013] The suitable pharmaceutical carrier or diluant as well as all the other adjuvants possibly present in said pharmaceutical composition and the percentage of active compounds/ pharmaceutical carriers or diluants can be selected and adapted by the person skilled in the art according to the type of viral infection, to the type of viral strain (resistence or non-resistence to one or more antiviral compounds) and to the possible side-effects of said active compounds, adjuvants, carriers or diluants.

[0014] The present invention also related to the administration of said inhibitor with a sufficient level in the serum (in the viral infected patient) of other activating compounds (such as TNF, IL 18, IL-2 or another suitable natural or synthetic activator of the provirus transcription).

[0015] Such synergic effect results in the provirus activation.

[0016] The activation of HIV expression according to the invention combined with HAART treatment (combination of several drugs against HIV) thus leads to the reduction or elimination of said latently infected cells and preferably to the eradication of the HIV in the patient's cells and in the circulating fluids.

[0017] Preferably, the mammal patient's cell is selected from the group consisting of lymphocytes cells, monocyte cells, macrophage cells, astrocyte cells or other cells which can be infected by a virus especially a retrovirus, more preferably a HIV-virus (HIV-1 and or HIV-2)

[0018] The suitable deacetylase inhibitor used according to the invention is preferably selected from the group consisting of already known products, such as the valproïc acid (VPA), previously used in the treatment of epilepsy and bipolar disorders (Johannessen, 2000, Neurochem International Volume 37, p.103-110), the sodium butyrate (NaBut) and some of its analogues already used in the treatment of behaviours induced by beta-globine synthesis, such as anemia and beta-thalassemia (Perrine et al., 1993, N. Engl. J. Med. 328, p.81-86; Dover et al.,1994, Blood 84, p.:339-343; Sher et al., 1995, N.Engl. J. MED. 332, P.1606-1610; Collins et al., 1995, Blood 85, p.43-46), the compound MS-27-275, the compound FR99-1228, the compound trichostatin A (TSA) and/or the compound

trapoxin which are already used as potential anti-tumoral compounds (Jung M., 2001, Current Medicinal Chemistry 8, p.1505-1511; p.74-77; Archer and Hodin, 1999, Curr.Opin.Genet.Dev.9, p.171-174; Marks P.A. et al., 2001, Curr.Opin.Oncol. 13, p.477-483; Saito et al., 1999, PNAS 96, p.4592-4597; Nakajuma et al., 1998, Exp.Cell.Res. 241,p.126-133; Qiu et al., 1999, Br.J.Cancer 80, p.1252-1258; Richon et al., 1998, PNAS 95, P.3003-3007. Redner et al., 1999, Blood 94, p.417-428; Saunders et al., 1999, Cancer Res. 59, p.399-404; Warrel et al., 1998, J.Natl.Cancer Installation. 90, p.1621-1625).

[0019] The present invention will be described in detail in the following description, which is present as a non-limiting example of the present invention

Detailed description of the invention

Materials and methods

Cell culture

[0020] The U1 and SupT1 cell lines were obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The monocytic cell lines U937 (#85011440) and the HL60 (#98070106) were obtained from the European Collection of Cell Cultures (ECACC). All cell lines were grown as reported (Van Lint et al., 1994; Van Lint et al., 1997).

Plasmid constructs

[0021] A 790-bp fragment containing the HIV-1_{LAI} 5′ LTR (nt 1-789, numbering is according to the LAI provirus where nt +1 is the start of U3 in 5′ LTR) was prepared from pLTR-CAT (Van Lint et al., 1994) by digestion with *Pst*I, blunt ending of 3′ overhang with T4 DNA polymerase and

digestion with *Xba*I (successively), and this fragment was then cloned in pGL2-Basic (Promega) digested with *Acc65*I, blunted with Klenow polymerase and digested with *Nhe*I. The resulting plasmid was designated pLTR(1-789)-luc. To construct pLTR(1-789)mut κB-luc, pLTR(1-789)-luc was used as a substrate for mutagenesis of the two κB sites by the Quick Change Site-Directed Mutagenesis method (Stratagene). Mutations were generated with the following pair of mutagenic oligonucleotide primers (mutations are highlighted in bold and B sites are underlined on the coding strand primer): CV211/CV212: 5'-CGAGCTTGCTACA**ACTC**ACTTTCCGCTG**GTCA**CTTTCCAGGGAGG-3'. The mutated construct was fully resequenced after identification by cycle sequencing using the Thermosequenase DNA sequencing kit (Amersham).

[0022] A 497-bp fragment containing the HIV-1_{LAI} 5' LTR (nt 292-789) was prepared from pLTR-CAT by digestion with *Ava*I, blunt ending of 5' overhang with Klenow polymerase and digestion with *Xba*I successively, and this fragment was then cloned in pGL2-Basic digested with *Acc65*I, blunted with Klenow polymerase and digested with *Nhe*I. The resulting plasmid was designated pLTR(292-789)-luc.

[0023] The plasmids pLTR(1-789)-luc and pLTR(292-789) were digested with *Hind*III and religated, thereby resulting in the deletion of the region extending from nt 532 to 789 and generating pLTR(1-531)-luc and pLTR(292-531)-luc, respectively.

[0024] To construct pLTR(345-531)-luc, a 186-bp fragment containing the HIV-1_{LAI} 5' LTR (nt 345-531) was generated by PCR amplification of pLTR-CAT, digested with *Kpn*I (site added in the 5' primer) and *Hind*III (site added in the 3' primer), and cloned into the *Kpn*I-*Hind*III-

restricted vector pGL2Basic. The 5′ primer oligonucleotide encompassed the coding strand sequence from nt 344 to 377 and contained an added *Kpn*I restriction site (underlined) at the 5′ end (5′-CGGGGTACC^{nt344}TACAAGGGACTTTCCGCTGGGGACTTTCCAGGG-3′). The 3′ primer oligonucleotide encompassed the complementary sequence of the LTR from nt 505 to 534 and contained an added *Hind*III site (underlined) at the 5′ end (5′-AGGCAAG^{nt534}CTTTATTGAGGCTTAAGCAGTGGGTTCCC-3′). The same strategy was used to construct pLTR(345-531)mut B-luc except that the 5′ PCR primer contained mutations (indicated in bold) in the two κB sites (5′-CGGGGTACC^{nt344}TACAA**CTC**ACTTTCCGCTG**CTC**ACTTTCCAGGG-3′).

[0025] The pLTR(A, B, C1, D, E, F, G and AG)-luc were previously described (Jeeninga et al., 2000).

[0026] The plasmids pRSV-p50 and pRSV-p65 were obtained from Dr. Gary Nabel and Dr. Neil Perkins through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). To construct pRSV-p65ΔATG, pRSV-p65 was used as a substrate for mutagenesis of the p65 open reading frame by the Quick Change Site-Directed Mutagenesis method (Stratagene). Deletion of the ATG initiation codon was generated with the following pair of mutagenic oligonucleotide primers: CV269/CV270: 5′-GCACCTCCAAGCTTCACC**GACGAACTGTTCCCC**-3′ (the region highlighted in bold on the coding strand primer indicates the p65 open reading frame (aa 2 to 6) starting at the second amino acid). The mutated construct was fully resequenced after identification by cycle sequencing using the Thermosequenase DNA sequencing kit (Amersham).

Transient transfection and luciferase assays

[0027] SupT1 cells were transfected using the DEAE-dextran procedure as previously described (Van Lint et al.,

1994). At 20 h post-transfection, the cells were treated or mock-treated with TSA (450 nM when a single dose was used) (Sigma Chemical Co.), NaBut (5 mM) (Sigma Chemical Co.), TNF (10 ng/ml) (R&D Systems) or combination of these drugs. At 42 h post-transfection, cells were lysed and assayed for luciferase activity (Promega). Luciferase activities derived from the HIV-1 LTRs were normalized with respect to protein concentration using the Detergent-Compatible Protein Assay (Bio-Rad).

Electrophoretic mobility shift assays (EMSAs)

[0028] Nuclear extracts were prepared from nuclei by a rapid method described by Osborn (Osborn et al., 1989). All buffers contained the following protease inhibitors: antipain (10 μg/ml), aprotinin (2 μg/ml), chymostatin (10 μg/ml), leupeptin (1 μg/ml) and pepstatin (1 μg/ml). Protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine plasma gamma globulin as a standard. EMSAs with the HIV-1 NF-KB probe were performed as previously described (Van Lint et al., 1996a). Briefly, nuclear extracts (10 μg of protein) were first incubated at room temperature for 10 min in absence of probe (in 2 16 μl reaction mixture containing 10 μg of Dnase-free bovine serum albumin (Pharmacial, 6 μg of poly(dI-dC) (Pharmacial as non-specific competitor DNA, 1 mM dithiothreital, 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA and 10% (Vol/Vol) glycerol). The probe was then added for a 20 min incubation period before running of the reaction mixture on a 6% nondenaturing polyacrylamide gel. For supershift assays, monoclonal antibody against p52 (#05-361 Upstate Biotechnology), and polyclonal antibodies against p50 (#06-886 Upstate Biotechnology), p65 (sc-109X), RelB (sc-226X), c-rel (sc-

6955X) (Santa Cruz Biotechnology, Inc.) were added at a final concentration of 2 μg/reaction to the binding-reaction mixture at the end of the binding-reaction for an additional 30 min incubation at room temperature before electrophoresis. As loading controls, the same nuclear extracts were tested for binding of Oct1 to an Oct1 consensus probe (5'-TGTCGAATGCAAATCACTAGAA-3', SantaCruz Biotechnology, Inc).

RNase protection analysis

[0029] Total RNA samples were prepared using the commercial RNAqueous Phenol Free Total RNA Isolation Kit (Ambion) from $5x10^6$ cells treated or mock-treated with TSA or NaBut or/and TNF during 6 h. HIV-1-specific transcripts were detected by RNase protecion analysis (RPAII kit, Ambion). Reactions were carried out according to the manufacturer's recommendations and the bands were visualized by autoradiography. An HIV-1-specific ^{32}P-labeled antisense riboprobe was synthesized *in vitro* by transcription of *Xba*I-restricted pGEM23 (a gift from M. Laspia) with SP6 polymerase by standard methods (Promega). This HIV-1 antisense riboprobe protected two RNA fragments of 83 and 200 nt, which corresponded to the 5' and 3' LTR, respectively (Laspia et al., 1993). As control, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antisense probe was synthesized by the same method and used on the same RNA samples.

Western blot analysis

[0030] Nuclear and cytoplasmic extracts were prepared as previously described (Osborn et al., 1989 and Schoonbroodt et al., 2000, respectively). Proteins (50 μg) were boiled for 3 min, analyzed on SDS-10% polyacrylamide gels, and transferred to Immobilon-P membranes (Millipore,

Bedford, MA). Membranes were blocked by preincubation with 1% Western Blocking Reagent (Roche) in TBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), and probed for 90 min at room temperature with the following antibodies: IκB-alpha (#06-494, Upstate Biotechnology, 1:1000 dilution), IκB-beta (#sc-945, 1:1000), IκB-epsilon (#sc-7156, 1:1000) and p65 (#sc-109X, 1:1000) (Santa Cruz Biotechnology, Inc.). Membranes were then washed twice with 0.5% Western Blocking Reagent (Roche) in TBS, washed twice with TBST (TBS+0.1% Tween 20) and complexes were then detected by incubation for 30 min with a peroxidase-conjugated anti-mouse IgG/peroxidase-conjugated anti-rabbit IgG (#1520709, Roche, 1:1000 dilution). Membranes were washed with TBST, analyzed using Amersham's enhanced chemiluminescence system (Amersham Pharmacia Biotech., Aylesbury, U.K.), and exposed to X-ray film to visualize the bands.

Virus production assay

[0031] HIV-1 production was measured by determining p24 antigen secretion in culture supernatants by an enzyme-linked immunosorbent assay (ELISA) (Innogenetics).

Generation of viral stocks

[0032] A derivative of pILIC (a circularly permuted infectious molecular clone of NL4-3 HIV-1 kindly provided by Dr. A. Rabson) was previously constructed and referred to this construct as pHIV (Van Lint et al., 1997). HIV-1 infectious DNA was generated from this single-LTR-containing proviral construct by *BamHI* digestion and self-ligation. This cocatemerized proviral DNA (10 μg) was generated into 10^7 JEG.1 cells (a clonal line of Jurkat cells) by using the DEAE-dextran procedure. At 24 h post-transfection, the culture was cocultivated with 10^7 SupT1

cells to allow rapid and efficient recovery of progeny virus. Virus stocks were prepared from cell-free supernatants after filtration through a 0.45-μm-pore-size membrane at the peak of viral production (day 12 to 14 after transfection). Stocks were quantified by determining p24 concentration for subsequent infectivity studies.

Viral infections

[0033] Infections were carried out by incubating $0.5x10^6$ U937 cells with 50 ng of p24 of HIV-1 viral stock (at 37°C for 2 h in 500 μl of culture medium). After infection, the cells were pelleted at 300x*g*, washed three times with 1 ml of culture medium, resuspended in 1 ml of complete RPMI 1640 medium (Gibco-BRL, Life Technologies), and grown under standard conditions. One day after infection, the cells were treated or mock-treated with TSA or/and TNF. Every 2 days, aliquots of 200 μl were removed from the infected cultures and replaced by complete RPMI 1640 medium. The aliquots were assayed for p24 antigen concentration following centrifugation (300x*g*) in order to monitor the kinetics of viral replication.

RESULTS

TSA inducibility of different deleted HIV-1 LTRs

[0034] In order to delineate the LTR sequences responsible for activation of the HIV-1 promoter activity in response to TSA, a series of pLTR-luciferase reporter constructs containing various 5' and/or 3' deletions within the viral promoter region (the prototype LAI strain of HIV-1 subtype B) were generated. The five resulting plasmids were designated pLTR(1-789)-luc [containing the complete 5' LTR plus the leader region up to the beginning of the GAG open reading frame], pLTR(292-789)-luc, pLTR(1-531)-luc,

pLTR(292-531)-luc and pLTR(345-531)-luc, respectively (Figure 1A, coordinates with respect to the LAI provirus where nt +1 is the start of U3 in the 5' LTR). These plasmids were transiently transfected into the human CD4+ T-lymphoid cell line SupT1. Transfected cells were mock-treated or treated with increasing concentrations of TSA (0, 250, 500 and 1000 nM) and assayed for luciferase activity. Results presented in Figure 1B show the TSA fold inductions for each construct to eliminate the variations due to the differences in basal activity observed with the various deleted LTRs. All LTR constructs were activated by TSA in a dose-dependent manner. pLTR(1-789)-luc and pLTR(292-789)-luc were induced 13.8 to 27.0-fold and 10.9 to 27.6-fold, respectively. This induction by TSA is likely to be explained by histone hyperacetylation (Van Lint et al., 1996a; Van Lint et al., 1996b; Sheridan et al., 1997; Steger et al., 1998) and/or by acetylation/deacetylation phenomenons involved in the regulation of transcription factors binding to the LTR.

[0035] The constructs pLTR(1-531)-luc, pLTR(292-531)-luc and pLTR(345-531)-luc presented TSA fold activations from 3.51 to 17.2-fold, from 4.57 to 11.2-fold and from 5.14 to 11.6-fold, respectively (Figure 1B), indicating a decrease in TSA inducibility associated with the deletion of the 3' region encompassing nt 532 to 789. A possible explanation for this decrease in TSA inducibility would be the absence of the nucleosome nuc-1 in the 3' deleted LTR constructs when assembled into chromatin since an important part of the DNA sequence wrapped around nuc-1 was lacking in these constructs (Figure 1A). Even though we did not analyze in this study the chromatin organization of the LTR constructs after transient transfection, several studies have reported that the DEAE-dextran method used here allows an almost

native chromatin structure on the transiently transfected DNA templates (Jeong and Stein, 1994).

[0036] Importantly, a significant TSA inductibility was still observed with the smallest LTR (nt 345 to nt 531), containing the TATAbox, the 3 Sp1 sites, the LBP-1/YY1 site and the 2 κB sites (Figures 1A and 1B). This could be consistent with the recruitment at the level of these sites of different factors presenting linkages with acetylation/deacetylation phenomenons: $TAF_{II}250$ (Mizzen et al., 1996), TFIIE and TFIIF (Imhof et al., 1997), Sp1 (Billon et al., 1999; Doetzlhofer et al., 1999; Suzuki et al., 2000; Xiao et al., 2000), YY1 (Coull et al., 2000) and p65 (Perkins et al., 1997; Gerritsen et al., 1997).

Intact KB sites are required for maximal TSA inducibility of the HIV-1 promoter

[0037] The NF-KB binding sites of the HIV-1 proximal enhancer region confer a high rate of transcription to the viral promoter in activated T-cells and monocytes/macrophages (Nabel and Baltimore, 1987; Griffin et al., 1989; Osborn et al., 1989). In order to investigate the functional role of these κB sites in the inducibility of the LTR by TSA, transient transfection experiments were performed into the SupT1 cell line with LTR luciferase reporter plasmids that contained or not point mutations in the two κB sites (pLTR(345-531)mut-kB-luc or pLTR(345-531)-luc, respectively). SupT1 cells were then mock-treated or treated with increasing concentrations of TSA (from 31 to 3000 nM) and assayed for luciferase activity. Up to a concentration of 500 nM in TSA, we observed similar fold-inductions with both the wild-type and the mutated LTR (Figure 2A). In contrast, at TSA concentrations higher than 500 nM, the wild-type LTR further responded to TSA in a

dose-dependent manner (up to 61-fold induction at 3000 nM in TSA), whereas the mutated LTR reached a plateau (with a maximal 27-fold induction at 500 nM in TSA) (Figure 2A). Importantly, similar results were obtained when comparing the TSA inducibility of the full-length LTR and its NF-KB mutated homologue (pLTR(1-789)-luc and pLTR(1-789)mut κB-luc, respectively) (Figure 2B), indicating that the observations did not result from an artificial regulation of the reduced LTR.

[0038] Thus, these results demonstrate a regulatory link between the full responsiveness of the HIV-1 LTR to TSA stimulation and the presence of intact κB sites in the viral enhancer.

Synergistic activation of HIV-1 promoter activity by NF-KB and TSA

[0039] To further investigate this link between NF-KB and TSA, SupT1 cells were transiently cotransfected with the pLTR(345-531)-luc construct and with increasing amounts (from 6ng/6ng to 1600ng/1600ng) of expression vectors for p50 and p65 (pRSV-p50/pRSV-p65). Cells were treated with TSA (450 nM) or mock-treated and assayed for luciferase activity (Table 1). As expected, in the absence of TSA, p50/p65 trans-activated the HIV-1 promoter in a dose-dependent manner up to 7.66-fold (Table 1, p50/p65-fold activation, lanes 2 to 13). No transactivation was observed when a control expression vector for p65ΔATG was used in the cotransfection assays. Treatment of cells with TSA alone resulted in a 51.8-fold activation of transcription (Table 1, lane 1). Remarkably, when cells were both cotransfected with increasing amounts of expression vectors for p50/p65 and treated with TSA, a strong synergy was observed between the two activators, resulting in

transactivations ranging from 95.0- to 2655-fold (Table 1, p50/p65+TSA-fold activation, lanes 2 to 13). Transcriptional activators synergize when their combination produces a transcriptional rate that is greater than the sum of the effects produced by the individual activators. Transfection of 1200 ng/1200 ng of pRSV-p50/pRSV-p65 led to a 7.38-fold stimulation of transcription in absence of TSA, whereas, in presence of TSA, it led to a 2655-fold stimulation (Table 1, lane 11). This amount of transcription is 45-fold greater (fold synergism) than the sum of the effects produced by each activator separately (51.8+7.38). Similarly, transfection of 600 ng/600 ng of pRSV-p50/pRSV-p65 in presence of TSA stimulated transcription 1590-fold, corresponding to a 28-fold synergism (Table 1, lane 8). This synergism between p50/p65 and TSA persisted even at saturating amounts of p50/p65 proteins (see 1400ng/1400ng and 1600ng/1600ng of cotransfected p50/p65 plasmid DNAs), indicating that the observed effect was not the consequence of increased p50/p65 expression due to activation of the RSV promoter by TSA.

[0040] Synergistic activation by ectopically expressed p50/p65 and TSA required intact NF-KB binding sites in the HIV-1 proximal enhancer, since point mutations in these sites abrogated this effect (Table 1, pLTR(345-531)mut κB-luc, lanes 14 to 26). This implies that the synergistic effect was mediated by interactions at the B sites and not at the otherwise intact LTR(345-531) DNA sequences. RNase protection analysis, using an HIV-1 promoter-specific probe confirms and a luciferase gene-specific probe, that the synergism between p50/p65 and TSA occurred at the level of transcription.

[0041] In conclusion, these results demonstrate that TSA synergistically enhanced NF-KB-dependent transcriptional activation of the HIV-1 promoter, suggesting that the NF-KB signaling pathway can be functionally regulated by posttranslational acetylation *in vivo*.

Synergistic activation of HIV-1 promoter activity by cytokine TNF and deacetylase inhibitors

[0042] Pro-inflammatory cytokine TNF stimulates the HIV-1 LTR through activation of NF-KB in both human CD4+ T cells and monocytes/macrophages (Osborn et al., 1989; Duh et al., 1989). To determine whether the synergism between NF-KB and TSA could be observed in the context of a physiological activation of the NF-KB pathway, we examined the effect of TSA on the TNF-induced HIV-1 promoter activity. To this end, two human cell lines representative of the two major cellular targets for HIV-1 infection [the SupT1 cell line (a CD4+ T-lymphoid cell line) and the HL60 cell line (a CD4+ promonocytic cell line), Figure 3 were transiently transfected with the reporter constructs pLTR(345-531)-luc or pLTR(345-531)mut κB-luc. Transfected cells were subsequently mock-treated or treated either with TNF alone, either with TSA alone, or with both activators. Treatment of SupT1 cells with TSA alone or TNF alone resulted in a 43.5- or a 7.61-fold increase, respectively, of luciferase gene expression driven by the wild-type LTR (Figure 3). Remarkably, when cells were treated with both activators in combination, a 287-fold increase of luciferase expression above the control level obtained in absence of any treatment is observed, demonstrating an important synergism between TNF and TSA. Mutation in the κB sites (pLTR(345-531)mut κB-luc) abrogated both the activation of the LTR by TNF and its synergistic activation

by TSA and TNF (Figure 3). Similar results were obtained in transient transfection assays into the promonocytic cell line HL60.

[0043] In order to extend said observations to other deacetylase inhibitors, trapoxin and sodium butyrate (NaBut) was used in similar transfection assays (Figure 3). Treatment of SupT1 cells with NaBut alone resulted in a 27.2-fold increase in luciferase activity above the control level obtained in absence of any treatment, whereas treatment with NaBut and TNF in combination led to a 261-fold increase, indicating a strong synergy between the two activators (Figure 3). Again, intact κB sites were necessary for this synergism since their mutation (pLTR(345-531)mut κB-luc) abrogated both the activation of the LTR by TNF and its synergistic activation by TNF+NaBut (Figure 3). Similar results were obtained in transient transfection assays into the HL60 cell line.

[0044] In conclusion, these functional results demonstrate that the deacetylase inhibitors TSA and NaBut functionally synergized with TNF to activate the HIV-1 LTR. The synergism observed between TSA (NaBut) and TNF was strictly dependent on the presence of intact κB sites in the HIV-1 proximal enhancer.

Synergistic activation by TSA and TNF of LTR activity from the HIV-1 subtypes A through G of the major group M

[0045] HIV-1 isolates have been classified into three genetic groups: the major group (M), the outlier group (O) and the non-M, non-O group (N). All groups are thought to have arisen from independent zoonotic transmissions. The group M isolates that are responsible for more than 99% of all infections, have diversified during their worldwide spread. These isolates have been grouped according to their

genomic sequences and can be divided into 10 distinct subtypes termed A through J (Kuiken et al., 2000). The number of B sites differs among the subtypes. The prototypic subtype B (used in the above experiments), present in Europe and North America and studied most extensively in laboratories, contains two functional κB sites. Although there is some variation in the exact position and in the sequence of these sites, two similar κB sites are present in the LTRs of most described HIV-1 isolates, including isolates from subtypes A, D, F, G and AG (a recombinant between subtypes A and G). On the other hand, subtype C viruses generally contain three κB sites, whereas subtype E viruses contain one functional κB site (Montano et al., 1997).

[0046] To examine the impact of these differences among the various HIV-1 group M subtypes in the number, position and sequence of the κB sites, transient transfections of SupT1 cells with reporter luciferase constructs containing LTRs from subtypes A, B, C1, D, E, F, G and AG (Jeeninga et al., 2000), were assayed for their responsiveness to either TSA alone, either TNF alone or both in combination. Results presented in Figure 4 show the fold inductions for each subtype (obtained by dividing the luciferase activities of subtype X by the basal activity of this same subtype X) in order to eliminate subtype-specific differences in basal activity of the LTRs.

[0047] LTR activity of each subtype tested was induced by TNF alone from 2.55- to 6.63-fold and by TSA alone from 26.2- to 63.6-fold depending on the subtype. (Figure 4). Importantly, TSA+TNF together synergized to activate all the subtype LTRs. Subtype E, containing one κB site, was induced 169-fold by TNF+TSA, corresponding to a 3.7-fold synergism. The subtypes A, B, D, F, G and AG, containing

two κB sites, presented inductions from 216- to 596-fold, corresponding to synergisms from 5.1- to 11-fold. Subtype C1, containing three κB sites, was activated 802-fold by TNF+TSA, corresponding to a 11.8-fold synergism, a synergism 3 times higher than that observed for subtype E (Figure 4).

[0048] The viral promoter synergistic transcriptional activation by TNF+TSA is a common feature of HIV-1 subtypes A through G and a certain positive correlation exists between the number of κB sites present in the respective LTRs and the amplitude of the synergism between TNF and TSA.

Deacetylase inhibitors TSA and NaBut prolong TNF-induced NF-κB binding to DNA

[0049] To examine the effect of deacetylase inhibitors on NF-KB binding to DNA, Electrophoretic Mobility Shift Assays (EMSAs) were performed by using as probe an oligonucleotide corresponding to the two κB sites from the HIV-1 subtype B, LAI (Van Lint et al., 1996a). This probe was incubated with nuclear extracts prepared from SupT1 cells either mock-treated or treated with TSA, TNF, NaBut, TNF+TSA or TNF+NaBut for different periods of time (30 min, 1 h, 2 h and 4 h) (Figure 5). As expected, a rapid appearance of NF-KB binding activity was observed in response to TNF (Figure 5A, lane 3). Competition EMSAs shows that the two TNF-induced retarded complexes were NF-KB-specific and by supershift assays using antibodies directed against individual members of the NF-KB family that these two retarded complexes corresponded to p50/p65 heterodimers and to p65/p65 homodimers, respectively (Figure 5B). NF-KB appeared after a 30-min treatment and

faded away after a 1 h treatment (Figure 5A, lane 9). Treatment of cells with TSA alone or NaBut alone caused no induction of NF-KB binding activity even after a 4 h treatment (Figure 5A, lanes 2-8-14-20 or lanes 4-10-16-22, respectively). A 30 min treatment with TNF+TSA or TNF+NaBut caused an induction of NF-KB binding activity identical to that obtained with TNF alone (Figure 5A, lanes 5 or 6, respectively). Remarkably, induction by TNF alone compared with the inductions by TNF+TSA or TNF+NaBut at later times (1, 2, 4 h), NF-KB binding activity was prolonged up to 4 h in presence of TNF+TSA or TNF+NaBut (Figure 5A, compare lane 9 with 11-12, lane 15 with 17-18, lane 21 with 23-24). This finding indicated a sustained NF-KB binding to DNA after TNF+TSA or TNF+NaBut versus TNF treatment. Additionally, supershift analysis of the NF-KB complexes at the different activation times and conditions demonstrated no change in the dimer composition of the two retarded NF-KB complexes. TSA did not alter the binding of the constitutively expressed Oct1 transcription factor in either the presence or absence of TNF (Figure 5A, lower panel).

[0050] Taken together, these *in vitro* binding studies demonstrate that deacetylase inhibitors TSA and NaBut prolonged TNF-induced NF-KB binding to DNA but did not themselves stimulate NF-KB binding.

The presence of p65 is sustained in the nuclei of TNF-stimulated SupT1 cells in response to TSA or NaBut

[0051] The same nuclear extracts used in EMSAs were also examined by Western blotting with an anti-p65 antibody in order to follow the presence of p65 as a function of time in the nucleus after treatment with TSA, TNF, NaBut, TNF+TSA or TNF+NaBut. Immunoblotting revealed sustained

nuclear p65 expression after TSA(NaBut)+TNF versus TNF treatment (Figure 5C).

[0052] These results indicate that the prolonged NF-KB binding to DNA we observed after TNF+TSA(NaBut) versus TNF treatment (Figure 5A) coincided with a prolonged intranuclear presence of p65.

Delay in cytoplasmic IκB-alpha recovery in response to TNF+TSA versus TNF treatment

[0053] The nuclear expression and action of NF-KB requires signal-coupled phosphorylation and degradation of the IκB inhibitors, which normally bind and sequester NF-KB in the cytoplasm. The activation of *de novo* IκB-alpha gene expression by NF-KB likely plays a key role in the termination of nuclear NF-KB action, thereby ensuring a transient NF-KB transcriptional response. Therefore, we reasoned that the prolonged nuclear binding activity and presence of NF-KB we observed in response to TNF+TSA versus TNF treatment could result from a delay in cytoplasmic IKB-alpha recovery.

[0054] To test this hypothesis, the presence of the IKBs as a function of time in the cytoplasm after treatment with TSA, TNF or TNF+TSA was followed. Cytoplasmic extracts were prepared from SupT1 cells treated with these activators for different periods of time (30 min, 1 h, 2 h, 4 h) and analyzed for IKB-alpha, IKB-beta and IKB-epsilon expression by Western blotting. TSA alone did not induce IKB-alpha degradation (Figure 6, lanes 2, 6, 10 and 14). As expected, TNF induced a rapid degradation of the IKB-alpha protein (Figure 6, lane 3) followed by its recovery, which was completed 1 h after stimulation (Figure 6, lane 7). After TNF+TSA treatment, rapid IKB-alpha degradation

was also observed (Figure 6, lane 4), but in contrast to what we saw with TNF alone, its recovery was delayed up to more than two hours (Figure 6, compare lane 7 with 8 and lane 11 with 12). No change in IKB-beta and IKB-epsilon cytoplasmic concentration was observed after any treatment. Similar results were obtained when examining the combined effect of TNF and NaBut.

[0055] A marked delay in the recovery of the cytoplasmic NF-KB inhibitor, IKB-alpha, after TNF+TSA versus TNF treatment is correlated temporally with the sustained NF-KB binding activity and the sustained intranuclear presence of p65 that we observed after TNF+TSA versus TNF treatment by EMSAs and immunoblotting, respectively. This delay could thus explain the strong transcriptional synergism we observed between NF-KB and TSA on the HIV-1 promoter.

Synergistic activation by TSA and TNF of HIV-1 replication following infection of U937 cells

[0056] To assess the biological relevance of the TNF/TSA synergism, the effects of these drugs was tested on viral replication in the context of an infection by HIV-1. Infected U937 monocytic cells with a HIV-1 NL4-3 stock were mock-treated or treated with either TSA, either TNF or both activators. HIV-1 replication was monitored by measuring the production of p24 gag antigen in the cell supernatants over a 15-day period (Figure 7). Results indicated that, in absence of any treatment, infection resulted in progressive virus production. Following treatment with TSA alone or TNF alone, HIV-1 NL4-3 replicated more efficiently with levels of virus production higher than the control level. Importantly, TNF+TSA together synergized to enhance virus production at each time point. At day 15, TSA alone, TNF

alone and TNF+TSA increased p24 levels by 2-fold, 3-fold and 8-fold, respectively, above the control level obtained in absence of any treatment.

[0057] These data indicate that TNF and TSA synergistically increased the replicative capacity of the HIV-1 NL4-3 virus in U937 cells. These results were confirmed in three independent infection experiments performed in triplicate and were consistent with the results of the LTR-luciferase assays.

[0058] Thus, while the transcriptional activation of the HIV-1 promoter in response to TSA had been previously demonstrated in *ex vivo* transiently or stably transfected HIV LTR reporter constructs (el Kharroubi et al., 1998; Kiernan et al., 1999; Jordan et al., 2001), in latently HIV-infected cell lines (Van Lint et al., 1996a) and on *in vitro* chromatin-reconstituted HIV-1 templates (Sheridan et al., 1997; Steger et al., 1998), the results presented here constitute the first demonstration of the activating effect of a deacetylase inhibitor in the context of a natural HIV-1 infection. A synergistic effect of TSA and TNF on the level of HIV-1 replication as demonstrated.

Synergistic activation of HIV-1 transcription and replication by deacetylase inhibitors and TNF in latently infected cells

[0059] Different culture systems have served as *in vitro* models for post-integration latency, and the study of these cells has provided important insight into the mechanism of transcriptional reactivation and pathogenesis of HIV. The U1 monocytic cell line (cloned from a population of chronically HIV-1-infected U937 cells) is one of the most-studied models of post-integration latency. The inducing effect of TNF on endogenous HIV-1 replication in U1 cells has been correlated with the activation of NF-KB binding

to the viral enhancer and the stimulation of newly transcribed HIV-1 RNAs (Folks et al., 1987; Folks et al., 1988; Poli et al., 1990; Poli et al., 1994).

[0060] To study the effect of deacetylase inhibitors on HIV reactivation, the latently infected cell line U1 was treated for 24 h with TSA, TNF, NaBut, TNF+TSA or TNF+NaBut. Treatment with TSA alone, TNF alone or NaBut alone resulted in increases in p24 antigen release of 47.3, 30.3- and 118-fold, respectively, (Van Lint et al., 1996a) (Figure 8A). Induction by TNF+TSA and TNF+NaBut caused a 674- and 650-fold activation of virus production, respectively (Figure 8A). This synergistic activation by TSA (NaBut) and TNF of virus production in U1 cells took place at the transcriptional level. Indeed, RNAse protection analysis with an antisense riboprobe corresponding to the HIV LTR showed that treatment with TSA or NaBut resulted in a 4.3-fold or 6.3-fold increase of HIV-1 transcription, to a degree similar to that observed following TNF treatment (Figure 8B and 8C). After treatment with TNF+TSA and TNF+NaBut, a 42-fold and 48-fold induction of the steady-state HIV mRNA level, respectively, above the mRNA level was measured in the absence of any treatment. These data demonstrated a synergistic activation by TNF and deacetylase inhibitors of HIV-1 transcription in latently infected U1 cells. As an internal control, RNAse protection analysis of the same RNA samples using an antisense probe corresponding to the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed no change in the level of mRNA following treatment with any drug alone or in combination (Figure 8B). Moreover, Western blot analysis of cytoplasmic extracts from U1 cells treated with the different drugs revealed a delay in cytoplasmic IκB-alpha

recovery in response to TNF+TSA versus TNF treatment as observed in SupT1 cells (Figure 6 above).

[0061] These results demonstrate that the combination of TNF with a deacetylase inhibitor has a synergistic effect on reactivation of HIV-1 transcription and replication in the latently infected U1 cell line.

[0062] In summary, these results demonstrate a synergistic activation of the HIV-1 transcriptional promoter activity by NF-KB and inhibitors of deacetylases in transient transfection reporter assays and in acutely and latently infected cell lines.

[0063] The deletion analysis shows that the TSA inducibility of the HIV-1 promoter requires diverse sequences scattered throughout the LTR. This is in agreement with previous studies investigating the NaBut-mediated induction of LTR transcriptional activity (Laughlin et al., 1993). Nuc-1 is likely to be the nucleosome target of action of the deacetylases, since it is a nucleosome whose structure/conformation is affected, when deacetylases are inhibited. It is known that transfected DNA rapidly assembles into minichromosomes with histones attached (Kirkpatrick et al., 1994). Moreover, the DEAE-dextran transfection technique used here has been reported to allow the typical 160-bp DNA ladder characteristic of the physiological nucleosomal DNA (Jeong and Stein, 1994) and the *in vitro* chromatin-reconstituted HIV-1 templates corroborate the native nucleosomal organization (Sheridan et al., 1997).

[0064] Importantly, because of the numerous non-histone protein substrates for acetylation, the TSA response of the HIV-1 promoter could be explained for a large part by acetylation/deacetylation phenomenons involved in the regulation of nuclear factors binding to the LTR. On one

hand, several of these factors, including AP-1 (Zanger et al., 2001), ligand-bound nuclear hormone receptors (Minucci and Pelicci, 1999), c-Myb (Dai et al., 1996; Tomita et al., 2000), glucocorticoid receptor (GR) (Kamei et al., 1996), NF-AT (Avots et al., 1999), E-box binding proteins (McMahon et al., 2000), Ets-1 (Yang et al., 1998), TCF/LEF (Takemaru and (Hecht et al.,2000), NF-κB (Sheppard et al., 1999), Sp1 (Xiao et al., 2000), IRF (Masumi and Ozato, 2001) and the HIV trans-activator Tat (Benkirane et al., 1998) have been shown to interact with acetyltransferases.

[0065] On the other hand, several transcription factors that bind to the LTR, including unliganded nuclear hormone receptors (Minucci and Pelicci, 1999), GR (Ito et al., 2000), E-box binding proteins (Hassig et al., 1997), YY1 (Yang et al., 1996), Sp1 (Doetzlhofer et al., 1999), TCF/LEF (Billin et al., 2000) have been shown to interact with deacetylases. These factors therefore represent good candidates for the specific targeting of acetyltransferases and deacetylases to the HIV promoter, thereby regulating the acetylation level of histones (in particular nuc-1 histones) and/or transcription factor substrates binding to the LTR (such as c-Myb (Tomita et al., 2000), Sp1 (Suzuki et al., 2000), IRF (Masumi and Ozato, 2001), TFIIEK and TFIIF (Imhof et al., 1997) and Tat (Col et al., 2001). The addition and removal of acetyl groups on these histone and non-histone proteins could be crucial in controlling transcription initiation and elongation.

[0066] Thus, the HIV promoter appears to contain numerous *cis*-regulatory DNA elements involved in the inducibility of the LTR by TSA.

[0067] Importantly, a significant TSA inductibility was still observed with a reduced LTR (nt 345-531), containing the TATA box, the 3 Sp1 sites, the LBP-1/YY1

site and the 2 KB sites (Figure 1A and 1B). This was consistent with the recruitment at the level of these sites of different factors presenting linkages with deacetylation/acetylation processes:

1) κB sites: NF-KB-dependent gene expression requires the function of transcriptional coactivator proteins, including CBP/p300, P/CAF, SRC-1, which possess acetyltransferase activity (Sheppard et al., 1999). Moreover, there is some evidence to suggest that deacetylase inhibitors may function to positively regulate NF-KB transcriptional activity (Ito et al., 2000).

2) Sp1 sites: Sp1 is acetylated *in vitro* by p300 (Suzuki et al., 2000) and interacts with p300, which acts as a coactivator for Sp1-mediated transcriptional activation (Xiao et al.,2000). Sp1 has also been shown to interact directly with HDAC1 (Doetzlhofer et al., 1999).

3) TATA box: the general transcription factors TFIIEK and TFIIF are acetylated *in vitro* by P/CAF and p300 (Imhof et al., 1997). The TFIID subunit TAFII250 is a HAT (Mizzen et al., 1996).

4) LBP-1 site: LBP functions as a docking molecule for YY1, which in turn acts by recruiting HDAC1. This ternary complex represses the HIV-1 promoter, probably via the HDAC activity since this repression is blocked by TSA (Coull et al., 2000).

Potential functional role of the κB sites in the TSA inducibility of the HIV-1 LTR

[0068] By mutational analysis, a regulatory link between the full responsiveness of the HIV-1 LTR to TSA stimulation and the presence of intact KB sites in the viral enhancer was enhanced. Of note, up to a concentration

of 500 nM in TSA, the LTR with mutated KB sites (pLTR(345-531)mut KB-luc) responds to TSA equally well as the wild-type LTR (pLTR(345-531)-luc) (Figure 2). This could be explained by the presence of binding sites for transcription factors other than NF-KB, which are present in the nt +345/+531 region and linked to deacetylation/acetylation processes (TATA box, LBP, Sp1, see above). At TSA concentrations higher than 500 nM, the wild-type LTR was further induced, whereas the mutated-κB LTR was not. This differential TSA response between these two reporter constructs was observed in absence of TNF-induced transactivation of NF-KB or of ectopically expressed p50/p65. Therefore, this difference could result from the effect of TSA on NF-KB proteins present constitutively in the nuclei of SupT1 cells, although the majority of cellular NF-KB in unstimulated cells is cytoplasmic.

[0069] Mechanistically, in gel retardation assays TSA (or NaBut) prolongs TNF-induced NF-KB DNA-binding activity, whereas TSA alone (or NaBut alone) causes no induction of NF-KB. These *in vitro* binding studies coincided with a sustained nuclear p65 presence as revealed by immunoblotting. Importantly, Western blot analysis also revealed a marked delay in the cytoplasmic reappearance of the inhibitory protein IKB-alpha after TNF+TSA versus TNF treatment. This delay in IKB-alpha recovery correlated perfectly with the sustained nuclear binding activity and presence of NF-KB. These data therefore provide a molecular mechanism involving IKB-alpha for the functional synergism we observed between TNF and deacetylase inhibitors. IKB-alpha plays a pivotal role in the NF-KB signaling pathway. Indeed, the primary level of regulation

of NF-KB activity is through its retention in the cytoplasm through interactions with IKB-alpha. Moreover, the resynthesis of *de novo* IKB-alpha participates in a negative feedback system ensuring a transient NF-KB transcriptional response (reviewed in (Karin and Ben Neriah, 2000)).

[0070] Some proteins involved in the NF-KB/IKB signaling may have their expression and/or action modulated by TSA.

[0071] The molecular mechanisms mediating the TNF/TSA synergism are likely to be highly complex and to implicate phenomenons other than the delayed IκB-alpha recovery. On one hand, the direct acetylation of Rel family members could also intervene in the mechanism of synergistic activation by TNF and TSA. Two other groups have separately reported the interaction of p65 either with HDAC1 (Ashburner et al., 2001) or with HDAC3 (Chen et al., 2001b). These HDACs could repress expression of NF-KB-regulated genes by maintaining histones and/or other proteins in a deacetylated state. TSA or NaBut, which inhibit the HDAC activity, would increase NF-KB-dependent transcription by alleviating the chromatin- and/or factor-mediated block to transcriptional activation.

[0072] The institution of HAART has resulted in a major reduction of virus loads in individuals tolerating the regimen, a stabilization of the clinical course, and a significant decline in mortality/ morbidity (Hecht et al., 2000). Nonetheless, the persistence of HIV reservoirs (including latently-infected resting CD4+ memory T cells, persistently infected tissue macrophages, latently-infected naïve CD4+ and CD8+ T cells (Brooks et al., 2001), and possibly other still unknown reservoirs) has posed a sobering challenge to the long-term control or eradication

of HIV in infected individuals receiving HAART (reviewed in (Pierson et al., 2000). These latently infected cells are a permanent source for reactivation and lead to a rebound of viral load levels after interruption of HAART (Chun et al., 1997b).

[0073] Activators of HIV expression combined with HAART leads to the elimination of the latently infected cells and to the eradication of the infection. Indeed, it is likely that the latently infected cells die upon reactivation of virus (Perelson et al., 1997) and that HAART prevents spread of released virus to adjacent cells (Chun et al., 1998). It's important to note that an array of cytokines, including the proinflammatory cytokines TNF and IL-1 (inducers of NF-KB), are already copiously expressed in the microenvironment of the lymphoid tissues, which harbor latent viral reservoirs (Navikas et al., 1995).

[0074] Therefore, the results show that the use of deacetylases inhibitors in the treatment of HIV infection represents a valuable approach for purging the latently-infected reservoirs in HAART-treated individuals. These deacetylase inhibitors would synergize with the TNF already present at increased level in the serum of the HIV-infected individuals.

[0075] It is important to mention several points. First, these drugs do not present any cell-specificity. Second, this class of agents is safely administrated for other diseases including beta chain hemoglobinopathies such as beta-thalassemia and sickle cell anemia (Sher et al., 1995), and epilepsy and bipolar disorders (Phiel et al., 2001). Third, an increasing number of non-B HIV-1 subtype infections are currently diagnosed. In addition to the prototypical subtype B LTR, the LTRs from subtypes A through G of the HIV-1 group M were also activated

synergistically by TSA and TNF, and the amplitude of the synergism correlated with the number of κB sites in the respective LTRs, which varies from one (subtype E) to three (subtype C).

[0076] Overall, based on these results, the administration of deacetylase inhibitor(s) together with continuous HAART is proposed as a new therapeutic strategy to decrease in a subtype-nonspecific manner the pool of latent HIV reservoirs.

Table 1

	RLU -TSA	RLU +TSA	p50/p65 fold activation	p50/p65+TSA fold activation	Fold synergism
1 -	2.58	133	1.00	51.8	-
2 6/6	2.99	245	1.16	95.0	1.8
3 25/25	2.43	530	1.94	205	3.9
4 50/50	2.92	811	1.13	314	6.0
5 100/100	8.01	2961	3.11	1148	21
6 200/200	6.15	2723	2.39	1055	20
7 400/400	11.8	2631	4.59	1020	18
8 600/600	11.5	4102	4.45	1590	28
9 800/800	10.9	6129	4.24	2376	43
10 1000/1000	14.1	5809	5.46	2252	39
11 1200/1200	19.0	6851	7.38	2655	45
12 1400/1400	19.7	5394	7.66	2091	35
13 1600/1600	18.3	3189	7.10	1236	21
PLTR(345-531)mut KB-luc					
14 -	1.55	101	1.00	65.0	-
15 6/6	2.02	115	1.31	74.2	1.1
16 25/25	1.87	90.0	1.21	58.1	0.9
17 50/50	2.05	130	1.32	84.1	1.3
18 100/100	3.30	161	2.13	104	1.6
19 200/200	1.80	82.5	1.16	53.2	0.8
20 400/400	2.10	98.8	1.36	63.7	1.0
21 600/600	1.88	70.2	1.21	45.3	0.7
22 800/800	1.55	71.6	1.00	46.2	0.7
23 1000/1000	1.79	95.7	1.16	61.8	0.9
24 1200/1200	1.51	97.8	0.98	61.2	0.9
25 1400/1400	1.76	129	1.14	83.2	1.3
26 1600/1600	0.97	66.0	0.63	42.6	0.7

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CLAIMS

1. Use of a sufficient amount of deacetylase inhibitor combined with one or more compounds used in a viral treatment for the manufacture of a medicament for obtaining the elimination of integrated, functional and pathogenous viruses in a mammal cell, including a human cell.

2. Use according to the claim 1, wherein the compound(s) used in the viral treatment are compounds used in HAART treatment.

3. Use according to the claim 1, wherein said virus is a retrovirus.

4. Use according to the claim 3, wherein said retrovirus is a HIV-1 or HIV-2 virus.

5. Use according to any of the preceding claims, wherein the inhibitor of deacetylase and the compound used in the viral treatment, are combined in an adequate pharmaceutical carrier or diluant.

6. Use according to any of the preceding claims, wherein the deacetylase inhibitor(s) is selected from the group consisting of the valproïc acid (VPA), the sodium butyrate (NaBut), the compound MS-27-275, the compound FR90 1228, the trichostatin A (TSA), the trapoxin or a mixture thereof.

7. Use according to any of the preceding claims, wherein the mammal cell is selected from the group consisting of lymphocyte cells, monocyte cells, macrophage cells or astrocyte cells.

8. Use according to any of the preceding claims, wherein the sufficient amount of deacetylase inhibitor(s) is combined with one or more compounds selected from the group consisting of Tumor Necrosis Factor (TNF), Interleukin 18 (IL-18) and/ or interleukin 2 (IL-2).

9. A method for the elimination of integrated and functional and pathogenous viruses in a mammal cell of a patient, which comprises the step of administrating to said mammal patient or to said cell a sufficient amount of deacetylase inhibitor(s) combined with one or more compounds used in a viral treatment.

10. The method according to the claim 9, wherein the compound(s) used in the viral treatment are compounds used in a HAART treatment.

11. The method according to the claim 9, wherein said virus is a retrovirus.

12. The method according the claim 11, wherein said retrovirus is HIV-1 or HIV-2 virus.

13. The method according to any of the preceding claims 9 to 12, wherein the inhibitor of deacetylase and the compound(s) used in the viral treatment are combined in an adequate pharmaceutical carrier or diluant.

14. The method according to any of the preceding claims 9 to 13 wherein the deacetylase inhibitor is selected from the group consisting of valproïc acid (VPA), the sodium butyrate (NatBut); the compound MS-27-275, the compound FR90 1228, the trichostatin A (TSA), the trapoxin or a mixture thereof.

15. The method according to any of the preceding claims 9 to 14, wherein the mammal cell is selected from the group consisting of lymphocyte cells, monocyte cells, macrophage cells or astrocyte cells.

16. The method according to any of the preceding claims 9 to 15 , wherein the inhibitor(s) of deacetylase is combined with a compound selected from the group consisting of the Tumor Necrosis Factor (TNF), the Interleukin-18 (IL-18) or the interleukin-2 (IL-2).

17. A pharmaceutical composition comprising an adequate pharmaceutical carrier or a diluant, a deacetylase inhibitor and one or more compounds used in HAART treatment.

18. The pharmaceutical composition according to the claim 17, wherein said deacetylase inhibitor is selected from the group consisting of valproïc acid (VPA), the sodium butyrate (NaBut), the compound MS-27-275, the compound FR90 1228, the trichostatin A (TSA), the trapoxin or a mixture thereof.

19. The pharmaceutical composition according to the claims 17 or 18, which further comprises a compound selected from the group consisting of the Tumor Necros Factor (TNF), the Interleukin-18 (IL-18) and/ or the interleukin-2 (IL-2).

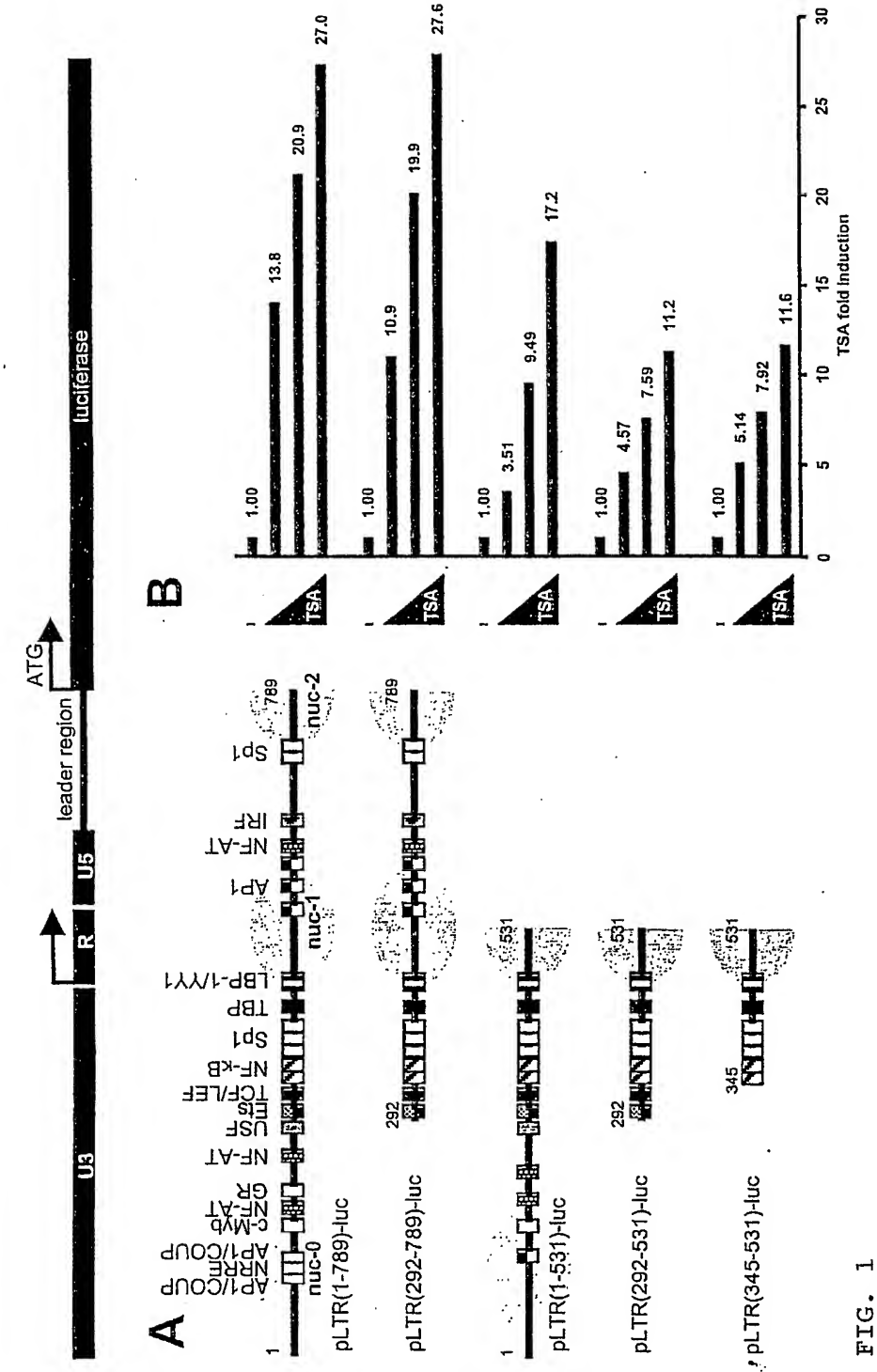
U3
R
U5
leader region
ATG
luciferase
A
B
pLTR(1-789)-luc
pLTR(292-789)-luc
pLTR(1-531)-luc
pLTR(292-531)-luc
pLTR(345-531)-luc
nuc-0
nuc-1
nuc-2
AP1/COUP
NZF
GR
GRE
c-Myb
NF-AT
TCF/LEF
NF-κB
Sp1
TBP
LBP-1/YY1
AP1
NF-AT
IRF
Sp1
1
292
345
531
789
1.00
13.8
20.9
27.0
1.00
10.9
19.9
27.6
1.00
3.51
9.49
17.2
1.00
4.57
7.59
11.2
1.00
5.14
7.92
11.6
TSA
0
5
10
15
20
25
30
TSA fold induction

FIG. 1

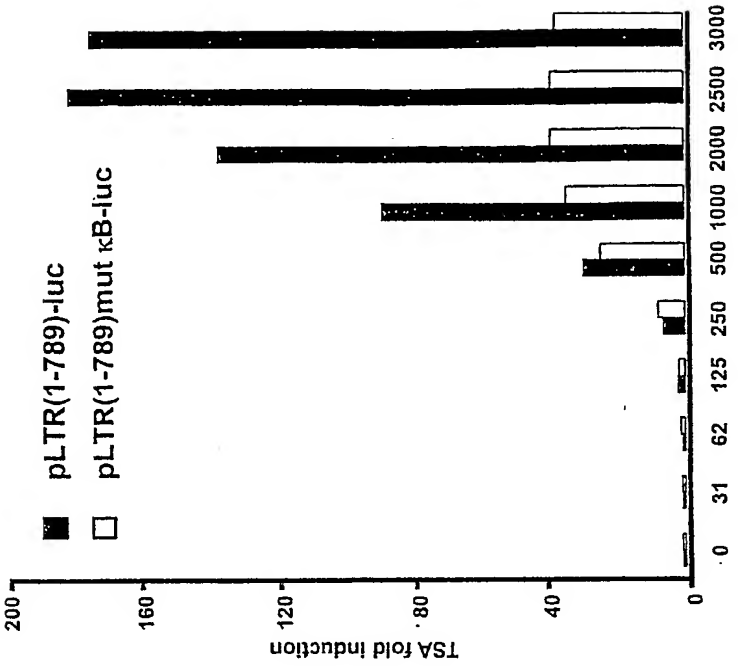
pLTR(1-789)-luc
pLTR(1-789)mut κB-luc
TSA fold induction
0
40
80
120
160
200
0
31
62
125
250
500
1000
2000
2500
3000
TSA (nM)

FIG. 2b

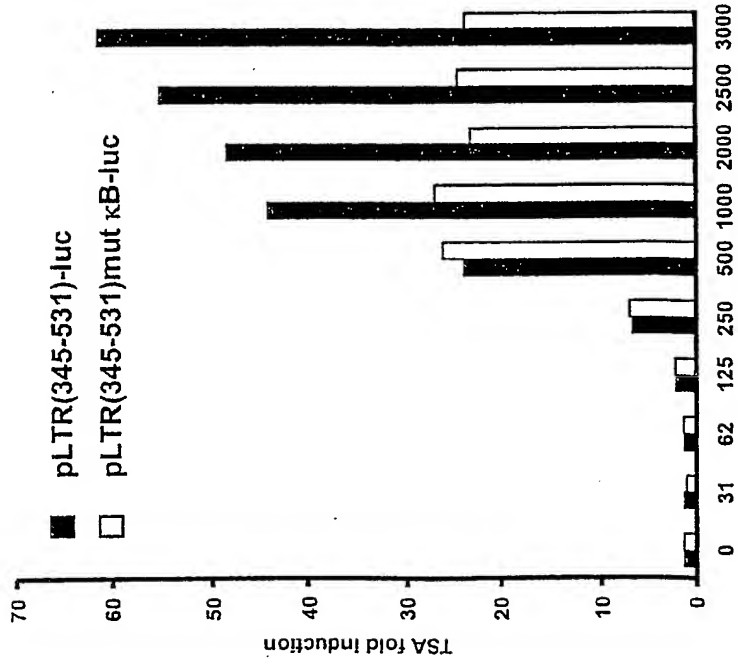
pLTR(345-531)-luc
pLTR(345-531)mut κB-luc
TSA fold induction
0
10
20
30
40
50
60
70
0
31
62
125
250
500
1000
2000
2500
3000
TSA (nM)

FIG. 2a

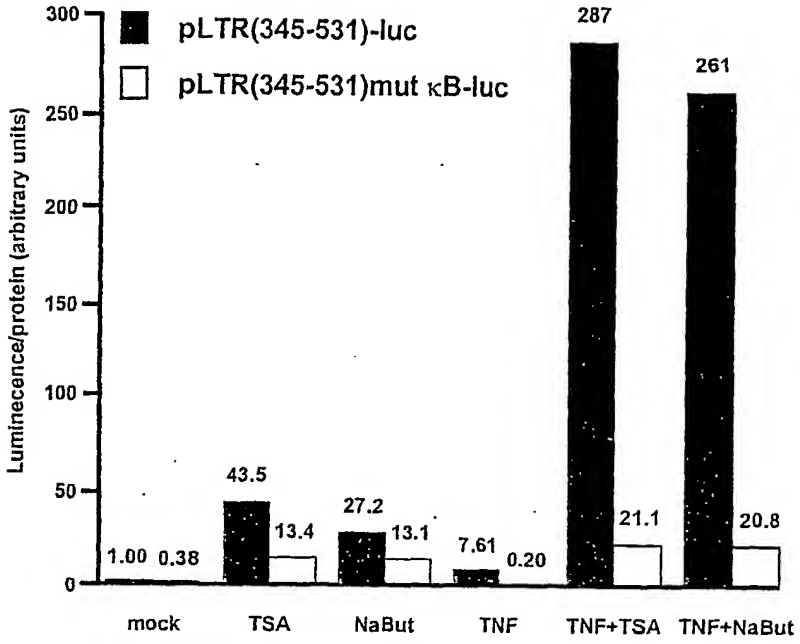
pLTR(345-531)-luc
pLTR(345-531)mut κB-luc
Luminecence/protein (arbitrary units)
300
250
200
150
100
50
0
1.00
0.38
43.5
13.4
27.2
13.1
7.61
0.20
287
21.1
261
20.8
mock
TSA
NaBut
TNF
TNF+TSA
TNF+NaBut

FIG. 3

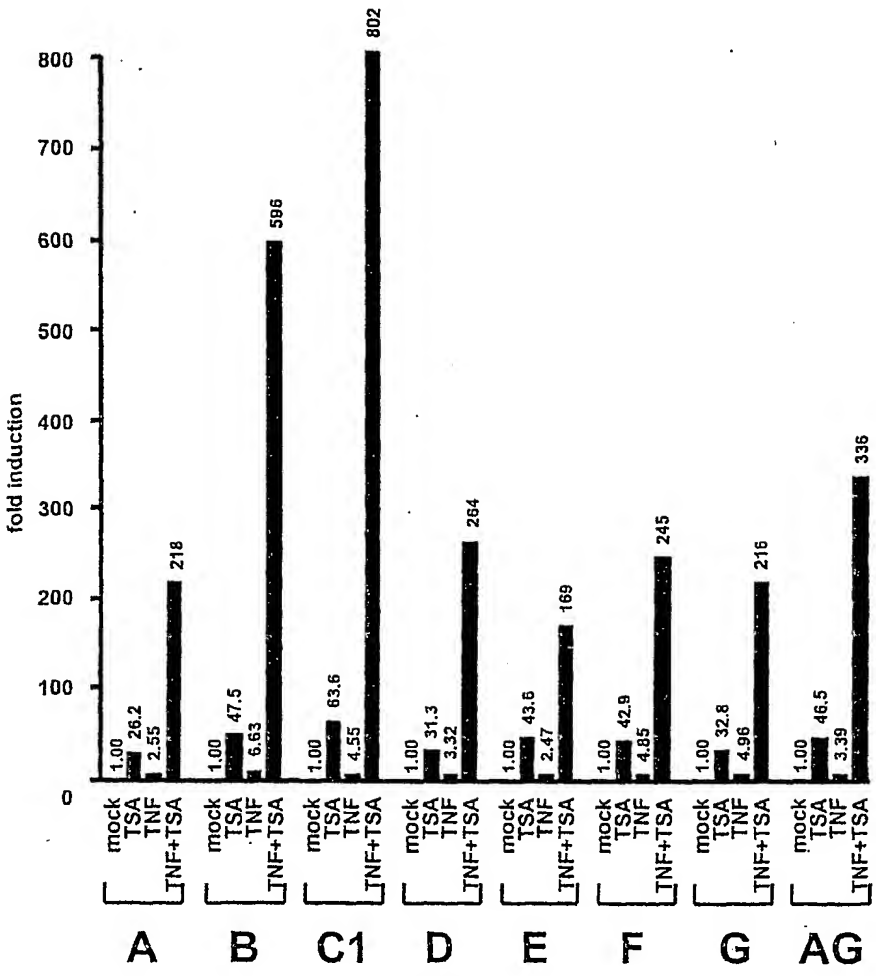
fold induction
800
700
600
500
400
300
200
100
0
1.00
26.2
2.55
218
1.00
47.5
6.63
596
1.00
63.6
4.55
802
1.00
31.3
3.32
264
1.00
43.6
2.47
169
1.00
42.9
4.85
245
1.00
32.8
4.96
216
1.00
46.5
3.39
336
mock
TSA
TNF
TNF+TSA
A
B
C1
D
E
F
G
AG

FIG.4

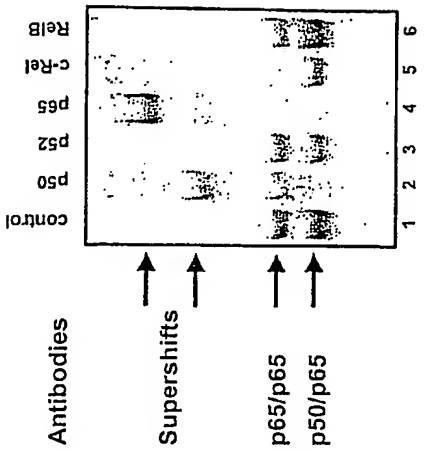

FIG. 5b

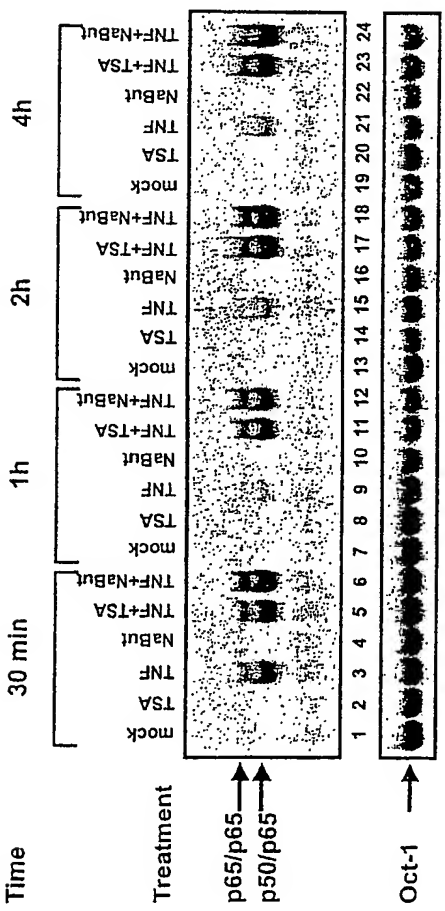

FIG. 5a

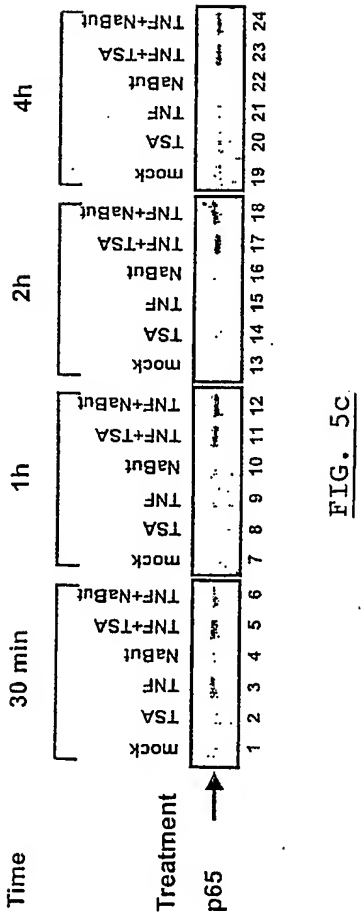

FIG. 5c

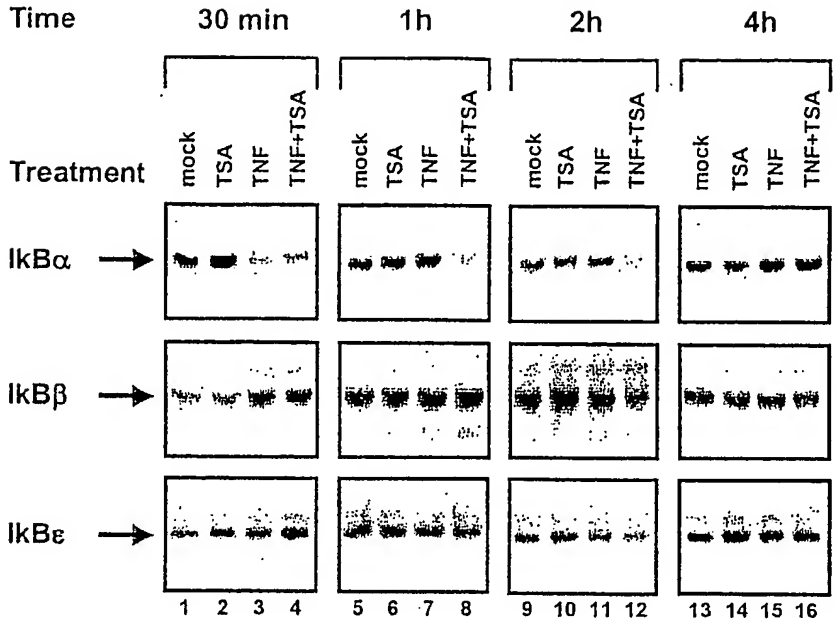
Time
30 min
1h
2h
4h
Treatment
mock
TSA
TNF
TNF+TSA
IkBα
IkBβ
IkBε
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 6

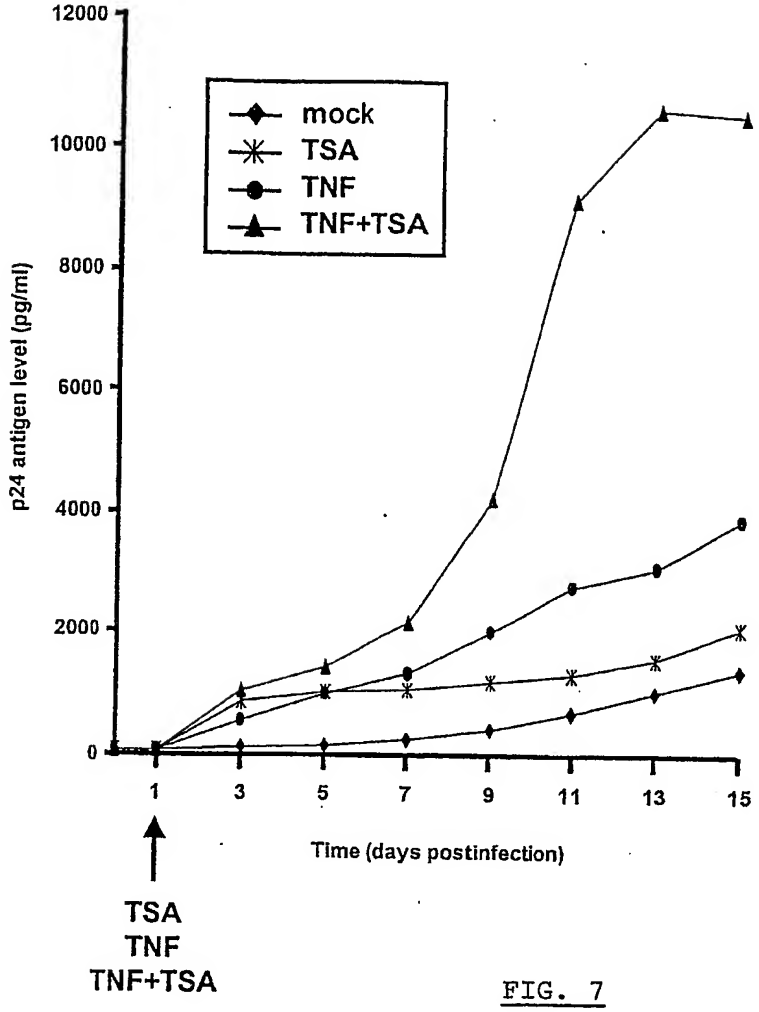
12000
10000
8000
6000
4000
2000
0
p24 antigen level (pg/ml)
mock
TSA
TNF
TNF+TSA
1
3
5
7
9
11
13
15
Time (days postinfection)
TSA
TNF
TNF+TSA

FIG. 7

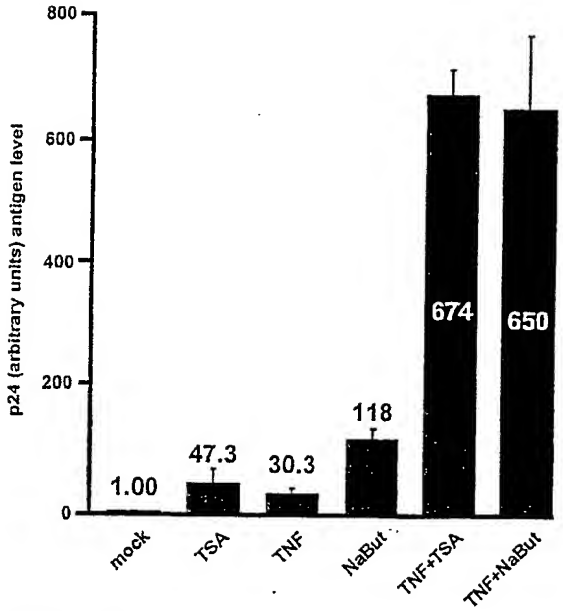

FIG. 8a

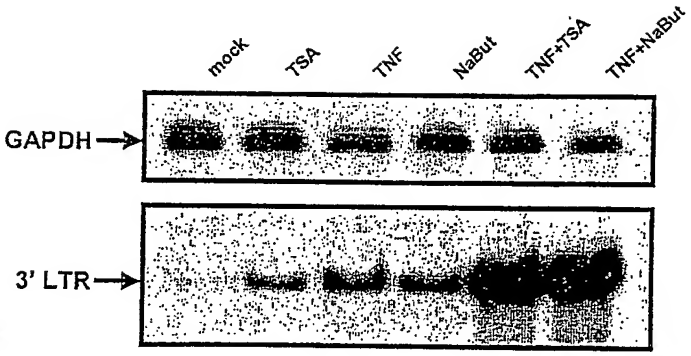

FIG. 8b

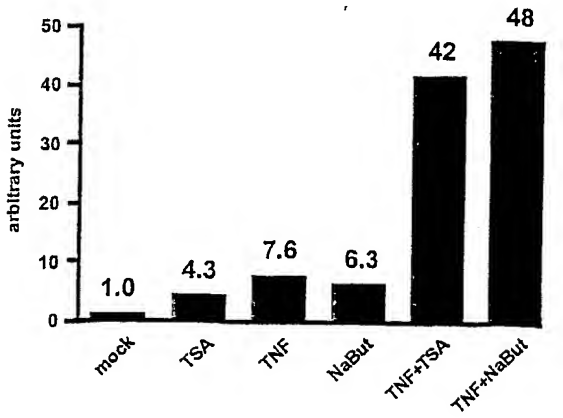

FIG. 8c

INTERNATIONAL SEARCH REPORT

Internatio pplication No
PCT/BE 02/00197

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K45/06 A61K38/19 A61K38/20 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, EMBASE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H.R.JENNINGS, F.ROMANELLI: "The use of valproic acid in HIV-positive patients" ANNALS OF PHARMACOTHERAPY, vol. 33, no. 10, 1999, pages 1113-1116, XP008015383 page 1113 page 1114, column 1 ---	1-7, 9-15,17, 18
X	M.A.LAUGHLIN E.A.: "Sodium butyrate treatment of cells latently infected with HIV-1 results in the expression of unspliced viral RNA" VIROLOGY, vol. 196, no. 2, 1993, pages 496-505, XP001149381 page 496 page 500, column 2 page 501, column 1 --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 March 2003	21/05/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Peeters, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	C-P. YAO E.A.: "Cytotoxicity induced by the combination of valproic acid and Tumor Necrosis Factor -alpha" BIOCHEMICAL PHARMACOLOGY, vol. 58, no. 3, 1999, pages 455-459, XP002203825 page 455 page 456, column 2 ---	1,5-9, 13-19
X	U.A.WALKER, N.VENHOFF: "Multiple mitochondrial DNA deletions and lactic acidosis in an HIV-infected patient under antiretroviral therapy" AIDS, vol. 15, no. 11, 2001, pages 1449-1450, XP008015381 page 1449 ---	1-7, 9-15,17, 18
X	B.HUG E.A.: "AntiretroviraleTherapie und Antiepileptika" SCHWEIZERISCHE MEDIZINISCHE WOCHENSCHRIFT, vol. 128, no. 29-30, 1998, pages 1138-1144, XP008015387 page 1138 page 1139, column 2 page 1140, column 1 page 1143 ---	1-7, 9-15,17, 18
X	B.RUSSELL: "An effect of anticonvulsants on antiretroviral therapy" JOURNAL OF NEUROVIROLOGY, vol. 4, no. 3, 1998, page 340 XP008015386 page 340 ---	1-7, 9-15,17, 18
X	P.PERRIN E.A.: "An Interleukin-2/sodium butyrate combination as immunotherapy for rat colon cancer peritoneal carcinomatosis" GASTROENTEROLOGY, vol. 107, no. 6, 1994, pages 1697-1708, XP008015385 page 1697 page 1705, column 2 ---	1,5-9, 13-19
X	M.KOVARIKOVA E.A.: "TNF-alpha modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line" EUROPEAN JOURNAL OF CANCER, vol. 36, no. 14, 2000, pages 1844-1852, XP001149811 page 1844 page 1847 page 1850 -----	1,5-9, 13-19

INTERNATIONAL SEARCH REPORT

Intern _ d application No.
PCT/BE 02/00197

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims relate to a product/compound/method defined by reference to a desirable characteristic or property, namely:
1) "Deacetylase inhibitor"
2) "Compound(s) used in a viral treatment"

The claims cover all products/compounds/methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products/compounds/methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product/compound/method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely claims 6,8,14,16,18,19, with due regard to the general idea underlying the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.